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Report

DELLA-Mediated Cotyledon Expansion Breaks Coat-Imposed Seed Dormancy

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Summary

Seed dormancy is a key adaptive trait in plants responsible for the soil seed bank. The long established hormone-balance theory describes the antagonistic roles of the dormancy promoting plant hormone abscisic acid (ABA), and the germination promoting hormone gibberellin (GA) in dormancy control [1–6]. Light, temperature, and other dormancy-breaking signals function to modulate the synthesis and perception of these hormones in the seed [7–10]. However, the way in which these hormones control dormancy in the imbibed seed remains unknown. Here, we show that the DELLA protein regulators of the GA response are required for dormancy and describe a model through which hormone signal integration and dormancy regulation is achieved. We demonstrate that cotyledon expansion precedes radicle emergence during *Arabidopsis* seed germination and that a striking correlation exists between final seedling cotyledon size and seed dormancy in the DELLA mutants. Furthermore, twelve previously characterized seed-dormancy mutants are also defective in the control of cotyledon size in a manner consistent with their effect on germination potential. We propose that DELLA-mediated, light-, temperature-, and hormone-responsive cotyledon expansion prior to radicle emergence overcomes dormancy imposed by the seed coat and underlies seed-dormancy control in *Arabidopsis*.

Results and Discussion

Despite the central role of dormancy control in the regulation of plant establishment, the mechanism through which dormancy (defined as quiescence that can be terminated by signals from the environment or by after-ripening) is broken in the imbibed seed has remained elusive. However, it is well established that the function of the plant hormones GA and ABA are central to the process [1–6]. GA signaling is mediated by the GA-promoted destabilization of the DELLA-protein growth repressors, predominantly RGL2 in *Arabidopsis* seeds [11–14]. In order to investigate the role of the DELLA proteins in dormancy control, we germinated freshly harvested seeds of DELLA single, double, triple, and quadruple mutants in the GA-deficient *ga1-3* background. In common with previous studies [11, 12, 14], we found that DELLA mutant combinations with a functional *RGL2* gene did not permit the germination of GA-deficient seed (although low levels of GA have been reported in *ga1-3*, these are insufficient to permit any germination of the *ga1-3* mutant). However, unlike the wild-type (Landsberg *erecta*) and *ga1-3*, both of which exhibited dormancy, seed with loss-of-function alleles for *RGL2*, *RGA*, and *GAI* were completely nondormant, even in the GA-deficient *ga1-3* background (Figure 1A). Therefore, we concluded that the GA signal transduction pathway regulates both dormancy breakage and germination in *Arabidopsis*. This conclusion is further supported by observations that simply nicking the seed coat promotes the germination of both dormant and GA-deficient seed, and such further emphasizes the role of GA in overcoming dormancy imposed by the seed coat in *Arabidopsis* [15]. Previously, it has often been concluded that GA has only a peripheral role in dormancy release [10, 16, 17], for instance, because applied GA cannot break the dormancy of strongly dormant seeds such as the *Arabidopsis* ecotype CVI (Cape Verde Island) [10]. In the light of our data, we suggest that integration of GA-derived signals by DELLA proteins is necessary to break dormancy but that GA alone is not sufficient and other factors must also be required.

Next, we investigated the role of the DELLA proteins in regulation of the seed response to after-ripening (loss of dormancy during dry storage). The sowing of the DELLA mutant seeds after increasing periods of storage showed that loss of function of RGL2 and RGA alone restored the dormancy of *ga1-3* seed to a wild-type level, whereas the combined loss of RGL2 and GAI was only slightly less effective (Figure 1A). Loss of RGL2 and RGA, or the combined loss of RGL2, GAI and RGL1 was sufficient to confer wild-type levels of germination on the *ga1-3* mutant, and these mutant seeds after-ripened at a rate similar to that of the wild-type. Notably, the germination of all DELLA mutant combinations containing *rgl2-1* and exhibiting dormancy at harvest was improved by storage, indicating that seed after-ripening functions to counter the inhibitory effects of RGL2, GAI, and RGA, whose combined loss of function leads to a non-dormant phenotype. The presence of the *ga1-3* allele shows that this effect is most likely independent of GA biosynthesis.

Seed after-ripening is known to reduce the accumulation of ABA in the seed after imbibition [10] so we promoted the germination of freshly harvested DELLA mutant seeds by cold treatment and tested the response to germination inhibition by exogenous ABA (Figure 1B).

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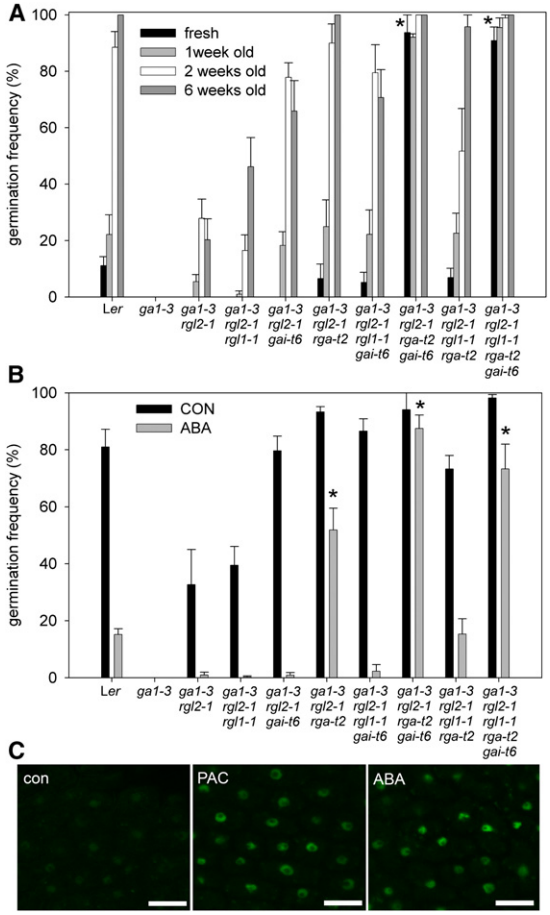

Figure 1. The Seed Germination of Wild-type, *ga1-3*, and DELLA Mutant Combinations in the *ga1-3* Background

Mutant combinations containing *ga1-3* but lacking *rgl2-1* did not germinate under any conditions [14] and are excluded from the presented data. Asterisks indicate lines with germination significantly greater than the wild-type at harvest (A) or after ABA treatment (B) ($p < 0.001$).

(A) The germination of freshly harvested seed and seed stored for the indicated period of time.

(B) The germination of cold stratified freshly harvested seed and stratified seed treated with 1 μM ABA on minimal water-agar medium. Data represent mean and standard error of the germination of five or six independent seed batches.

(C) pRGA:GFP-RGA fluorescence in 24-hr-imbibed seeds on water-agar media or media supplemented with 10 μM paclobutrazol or 10 μM ABA. Scale bars represent 10 μm.

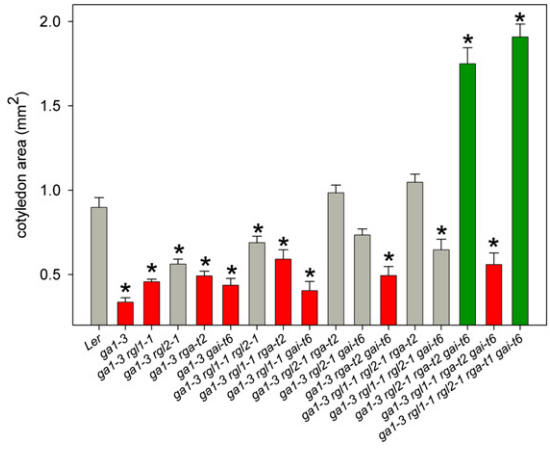

Figure 2. The Cotyledon Area of Wild-Type, *ga1-3*, and DELLA Mutants under Red Light

Data represent the mean and standard error of 12 cotyledons per genotype. Red bars indicate that seeds that do not germinate without exogenous GA [14], green bars indicate nondormant mutants, gray bars indicate mutants with similar dormancy to the corresponding wild-type (see Figure 1A). Asterisks indicate lines with a cotyledon size significantly different to that of wild-type ($p < 0.001$).

Low temperature strongly promoted the germination of DELLA mutant seeds containing *rgl2-1* in the presence of *ga1-3* (compare Figure 1A to Figure 1B), demonstrating that the DELLA proteins also likely regulate a GA-independent promotion of germination by temperature (in addition to the well-known GA-dependent promotion of germination by temperature [9]). Strikingly, seeds with loss of function of *RGL2*, *RGA*, and *GAI* exhibited strong ABA-resistant seed germination, whereas loss of *RGL2* and *RGA* was sufficient to confer a weak phenotype. Previously, it has been shown that RGA protein stability is increased by ABA in *Arabidopsis* root tissues, with transgenic plants expressing RGA under its own promoter with an N-terminal fusion to GFP [18]. We found that both the GA biosynthesis inhibitor paclobutrazol and ABA could enhance the stability of GFP-RGA in the cotyledons of nondormant seeds 24 hr after imbibition (Figures 1C–1E), demonstrating that RGA-protein abundance responds to both GA and ABA action in imbibed seeds. Thus, the DELLA proteins integrate GA and ABA signaling in the seed, and this mechanism parallels the integration of hormone signaling by DELLA proteins in vegetative tissues [18–20]. The DELLA proteins also therefore control germination in response to environmental cues that act through these phytohormones [8–10].

Our previous work had suggested that the germination and seedling photomorphogenesis-regulating bHLH transcription factor SPATULA (SPT) functions in part through GA action [21] so we tested the role of the DELLA proteins in the seedling response to red light. Although the DELLA proteins were found to be required for the GA regulation of hypocotyl elongation in *Arabidopsis*, we found no evidence that they were directly involved in the regulation of hypocotyl growth in response to light (see Figure S1 in the Supplemental Data available with this article online). However, we observed that GA and the DELLA proteins regulate cotyledon expansion in response to red light (Figure 2). The *ga1-3* mutant exhibited only limited cotyledon expansion, whereas the *ga1-3 rgl2-1 rga-t2 gai-t6* quadruple mutant and the *ga1-3 rgl2-1 rga-t2 gai-t6 rgl1-1* quintuple mutant both showed a highly significant increased cotyledon expansion phenotype compared to the wild-type. Strikingly, cotyledon expansion of the DELLA mutants correlated strongly with germination potential ($R^2 = 0.92$; Table S1): Nondormant combinations showed larger cotyledons than the wild-type; highly dormant genotypes showed decreased cotyledon size compared to the wild-type, and those combinations with roughly similar dormancy showed approximately wild-type cotyledon expansion. This effect was not an artifact of germination speed because nondormant or nicked seeds were used

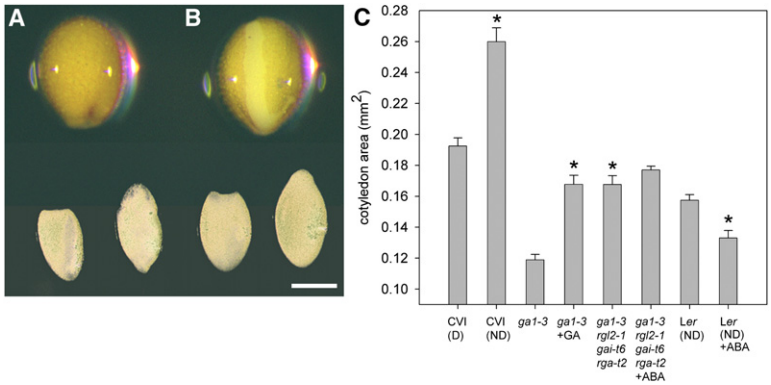

Figure 3. Cotyledon Size of 24-Hr-Imbibed Wild-Type CVI and *ga1-3* Seed prior to Radicle Emergence

(A) Dormant CVI seed and representative dissected cotyledons.

(B) Nondormant CVI seed and representative dissected cotyledons. The scale bar represents 200 μM.

(C) The cotyledon area of 24-hr-imbibed dormant (marked by the letter D) and nondormant (ND) imbibed CVI seed, *ga1-3* seed with and without the addition of 10 μM GA, and *ga1-3 rgl2-1 rga-t2 gai-t6* and L*er* with and without 10 μM ABA. Data represent mean and standard error of 12 cotyledons measured. Asterisks indicate data points significantly different from the respective control ($p < 0.001$).

to ensure an even germination rate, with any late germinating seedlings being actively excluded from the analysis. The finding that a strong correlation exists between the relative roles of the DELLA proteins in dormancy control and cotyledon-size control is not a general result of comparing the contribution of the various DELLA proteins to two DELLA-dependent processes: For instance, no significant correlation was observed between the relative contributions of the DELLA proteins in dormancy and hypocotyls-length control ($R^2 = 0.46$) or any other known DELLA-dependent processes (Table S1). This result showed that the DELLA proteins are required for the red-light control of cotyledon expansion and suggested a fundamental link between this response and the regulation of seed-dormancy breakage itself.

Seed germination in *Arabidopsis* is a distinctly biphasic process, the first phase of which is manifested by a longitudinal split in the seed coat along the embryo axis, and the second, radicle emergence through the micropylar endosperm [22]. In order to determine whether cotyledon growth could play a role in seed-dormancy breakage, we measured cotyledon size during the first phase, prior to radicle emergence (Figures 3A–3C). We found that compared to freshly harvested (dormant) CVI seed, the cotyledons of nondormant after-ripened CVI seed expanded an average of one third within the first 24 hr after imbibition and prior to radicle emergence. Cotyledon expansion of *ga1-3* required exogenous GA or the combined loss of RGL2, GAI, and RGA, underlining the role of GA and the DELLAs in regulating cotyledon expansion in the imbibed seed. Strikingly, ABA inhibited cotyledon growth in imbibed wild-type seeds ($p < 0.001$) but not in *ga1-3 rgl2-1 gai-t6 rga-t2* (Figure 3C), emphasizing both the key role of the DELLAs in mediating the ABA response in seeds and the way in which the GA and ABA regulation of cotyledon expansion reflects the GA and ABA control of seed dormancy itself (Figure 1). Furthermore, because mechanical damage to the seed coat is sufficient to terminate dormancy and overcome the germination requirement for GA in *Arabidopsis* [15] and many other species, we reasoned that cotyledon expansion within the seed leading to a breach in the seed coat would be sufficient to affect a similar result.

Our work suggested two hypotheses: firstly, that seed-dormancy breakage in the imbibed seed is regulated by cotyledon growth and secondly, that the mechanisms regulating cotyledon growth in the imbibed seed continue to be active in cotyledons during the seedling de-etiolation process. These hypotheses, if correct, suggested the powerful and previously unforeseen prediction that known seed-dormancy mutants should also be defective in the photomorphogenic control of cotyledon expansion in the seedling. Furthermore, these defects in cotyledon expansion should reflect the relative dormancy-enhancing or -reducing effects of the mutations in the imbibed seed. Therefore, we tested whether multiple previously characterized genes known to control seed dormancy also exhibited cotyledon-expansion phenotypes under red light.

We found that increased dormancy mutants such as those affected in light signal transduction [23] (*phyB-1*, *phyABDE* quadruple mutant), brassinosteroid synthesis and signaling [24] (*de-etiolated2* (*det2-1*), and *brassinosteroid insensitive 1* (*bri1-1*)), and peroxisomal β-oxidation [25] (*comatose* (*cts-2*) and the acyl-CoA oxidase double mutant *acx1-2 acx2-1*) all exhibited reduced cotyledon-expansion phenotypes compared to their corresponding wild-types (Figures 4A and 4B). Indeed, the small cotyledon phenotype of phytochrome mutants is well known, whereas the observation that brassinosteroid and peroxisomal β-oxidation are required for cotyledon expansion is novel. We also found that nondormant ABA-deficient and -insensitive mutants [4, 5] showed a large cotyledon phenotype. The ABA-insensitive mutants *abi1-1*, *abi2-1*, and *abi3-4* showed a large cotyledon phenotype compared to wild-types, whereas the *abi4-1* mutation that affects the ABA control of germination but not dormancy exhibited wild-type cotyledon size. That *abi3-4* mutants have an altered cotyledon ultrastructure in the seed has been previously documented [26]. Furthermore, *spt-10* shows decreased dormancy and displays a large cotyledon phenotype [21], whereas the related *pil5-1* mutant exhibits wild-type cotyledon size under red light (Figure 4C), consistent with the fact that it only shows a decreased dormancy phenotype in the dark [21]. The correlation coefficient comparing dormancy with cotyledon expansion in the seed-dormancy mutants and DELLA mutants combined was determined as 0.90, underlining the extremely close relationship between the control of the two phenomena. We also tested the response of cotyledon size to applied

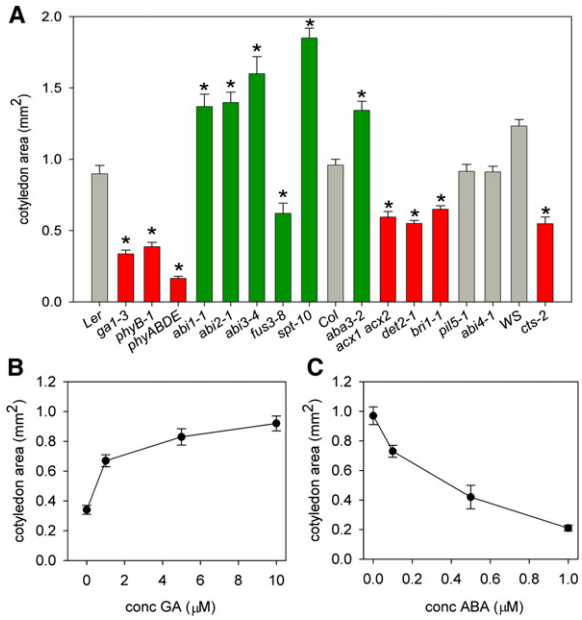

Figure 4. The Cotyledon Size of Mutants Affected in Seed-Dormancy Control

(A) Cotyledon area of 10-day-old *Arabidopsis* seed-dormancy mutants. Red bars indicate mutants with described increased dormancy phenotypes [23–25], and green bars indicate mutants with described reduced dormancy phenotypes [4, 5, 21]. Asterisks indicate lines with a cotyledon size significantly different to the wild-type ($p < 0.001$).

(B) The role of GA in the promotion of cotyledon expansion.

(C) The role of ABA in the inhibition of cotyledon expansion. Data represent the mean and standard error of 12 cotyledons measured.

GA and ABA. In a manner paralleling their affect on seed dormancy, applied GA promoted cotyledon expansion in 10-day-old seedlings, whereas applied ABA inhibited cotyledon expansion (Figures 4B and 4C).

Interestingly, the nondormant *fus3-8* mutant did not fit this pattern, with the very small *fus3-8* seeds giving rise to seedlings with correspondingly small cotyledons. Indeed, *fus3* mutant cotyledons are known to exhibit leaf-like qualities, such as the presence of trichomes [27]. Previous studies have shown that loss of *FUS3* has an additive effect on seed-specific gene expression in the *abi3* mutant background [28] and that FUS3 regulates an ABA- and GA-independent growth arrest early in seed development [28, 29]. *fus3* mutants also retain the ABA and GA sensitivity of seed germination [28]. It thus appears that loss of *FUS3* affects dormancy through a second parallel process, or that FUS3 is required for the initiation of dormancy during seed development, but does not play a role in the maintenance of dormancy in the imbibed seed.

We have shown that the DELLA proteins RGL2, RGA, and GAI regulate *Arabidopsis* seed dormancy and germination in response to GA and ABA action and environmental signals. *Arabidopsis* also contains a fifth uncharacterized DELLA protein, RGL3, which may also have a regulatory role in the seed. Our results also show that GA-, ABA-, and DELLA-controlled cotyledon growth occurs in imbibed nondormant seeds but not in dormant seeds. Furthermore, cotyledon expansion in seedlings is regulated by precisely those same genes that regulate seed dormancy. We therefore propose that a similar mechanism controls cotyledon expansion both before and after germination and that through cotyledon growth, GA and ABA and the DELLA proteins affect their regulation of dormancy breakage in the imbibed seed.

Experimental Procedures

Plant Material

The DELLA mutant combinations in the *ga1-3* background have been described [14] and were a gift from Jinrong Peng. The *ga1-3*, *aba3-2*, *abi1-1*, *abi2-1*, *abi3-4*, *abi4-1*, *det2-1*, *bri1-1*, *fus3-8* and *phyB-1* seeds were obtained from the Nottingham *Arabidopsis* Stock Centre. The *phyABDE* quadruple mutant has been described [30]. The *cts-2*, *acx1-2 acx2-1*, *pil5-1* and *spt-10* mutants have been described [21, 25].

Dormancy Assays

For each assay, mutant combinations were grown and harvested simultaneously in a greenhouse supplemented with white light so that a photoperiod is 16 hr. Seeds were sown on water-agar media and placed in continuous white light at (70–80 $\mu M \cdot m^{-2}$) at 22°C and germination scored after 7 days. Data shown represent the mean and standard error of the germination of 30–80 seeds from five or six independent seed batches per genotype. Stratification was for three nights at 4°C where indicated, and ABA (mixed isomers, Sigma, Poole, UK) was added at 1 μM concentration. Student's t test was used so that mutants whose dormancy differed significantly from wild-type could be determined.

Cotyledon Assays

Seedlings were grown at 20°C on MS medium under a red light LED array, and cotyledon areas were determined after 10 days of growth as described [21]. We added 20 mM glucose to ensure establishment of *acx1-2 acx2-1* and *cts-2* mutants. This does not affect dormancy [25]. Gibberellin (GA_3) was obtained from Sigma (Poole, UK). Nongerminating seeds were forced to germinate by nicking of the seed coat without damage to the seedling. Slow-germinating seedlings were excluded from the analysis for prevention of any influence of late germination on cotyledon size, and the late 10 day time point for the cotyledon measurements ensured the final cotyledon size for each mutant was obtained. For measuring the area of cotyledons prior to germination, seeds were imbibed on water-agar medium for 24 hr (with 10 μM GA or 10 μM ABA where indicated) and the seeds were manually dissected to reveal the cotyledons. The mean area of 20 cotyledons was determined per genotype per treatment. CVI seeds were stored for at least 3 months for dormancy breakage.

Confocal Detection of RGA-GFP

Confocal images of the upper surface of cotyledons from 24-hr-imbibed seeds on water-agar plates containing 10 μM ABA or 10 μM paclobutrazol (Greyhound Chromatography, Birkenhead, UK) where indicated were taken with a ×60 objective lens with an excitation wavelength of 488 nm. Images were obtained with a constant set of microscopic and image-intensity parameters with a Zeiss Axioplan upright confocal microscope.

Supplemental Data

Supplemental Data include Experimental Procedures, one figure, and one table and can be found with this article online at http://www.current-biology.com/cgi/content/full/16/23/2366/DC1/.

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